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SIMULTANEOUS QUANTITATION OF SALMONELLA SPECIES AND PSEUDOMONAS AERUGINOSA. I. POLLUTED WATERS. II. PERSISTENCE OF PATHOGENS IN SLUDGE TREATED SOILS. III. ANALYSIS OF WASTE TREATMENT SLUDGES FOR SALMONELLA SPECIES AS A SURVEILLANCE TOOL

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Environmental Protection Agency
Cincinnati, Ohio

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Simultaneous Quantitation of Salmonella Species and Pseudomonas Aeruginosa

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National Environmental Research Center
Cincinnati, Ohio**

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SIMULTANEOUS QUANTITATION OF SALMONELLA SPECIES
AND PSEUDOMONAS AERUGINOSA

- I. POLLUTED WATERS
- II. PERSISTENCE OF PATHOGENS IN SLUDGE TREATED SOILS *
- III. ANALYSIS OF WASTE TREATMENT SLUDGES FOR SALMONELLA SPECIES AS A SURVEILLANCE TOOL

by

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Waste Identification and Analysis Activities
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NATIONAL ENVIRONMENTAL RESEARCH CENTER
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- 1 -

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This report has been reviewed by the Water Quality Office, EPA, and approved for publication. Approval does not signify that the contents necessarily reflect the views and policies of the Environmental Protection Agency, nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

ABSTRACT

Simultaneous quantitation of Salmonella species and Pseudomonas aeruginosa. I. Polluted waters. Bernard A. Kenner

A practical sensitive quantitative method for the isolation and enumeration of Salmonellae and pyocyanogenic Ps. aeruginosa from wastewaters, treatment effluents, streams, stormwater runoff, and sludges, in all seasons of the year, is described.

The multiple tube test enrichment broth is enhanced by the addition of a killed culture of S. paratyphi A. Incubation of primary enrichment at 40 C is critical. Inoculum for secondary culture is taken from the surface of primary enrichment broth series as salmonella have a superior swimming ability. Secondary culture on non-toxic XLD agar plates allows the isolation of distinctive colonies of Salmonella sp. and Ps. aeruginosa, and the test as employed practically eradicates the time-wasting picking of false positive colonies due to related Enterobacteriaceae. Provisions are made to obviate MPN "skips".

The use of glass fiber ultra filters allows the testing of low turbidity to crystal-clear waters in a range from 1-liter to 10-gallon samples. The methods described are useful for testing treatment plant effluents, streams, well waters, and non-chlorinated drinking waters.

An appendix and six tables describe in detail media employed, schemata for five multiple tube methods, quantitative results obtained compared with published qualitative results, and proof for claims made for critical temperature, and the use of glass fiber ultra filters.

Research on practical methods for the quantitation of pathogenic bacteria which are important to health and water quality is an approved project of the National Environmental Research Center, Cincinnati.

II. Persistence of pathogens in sludge-treated soils. Bernard A. Kenner, G. Kenneth Dotson, and James E. Smith.

The persistence of Salmonella species, Pseudomonas aeruginosa, and fecal coliforms in soils treated with various types of municipal sludges is reported. The initial studies were made on sludge-treated soils in Pennsylvania under relatively mild spring and summer conditions. The second series of studies was made employing municipal sludges from Cincinnati sewage treatment plants and under more severe late fall and winter conditions.

From results obtained testing relatively small soil samples at weekly intervals, it is evident that sludge-borne pathogens may persist on agricultural or grass lands in the top two inches (5 cm) of soil for a least 21 weeks.

The use of unsterile municipal waste treatment sludges on land may well contribute to uninterrupted human and animal disease cycles.

III. Analysis of waste treatment sludges for Salmonella species as a surveillance tool. Bernard A. Kenner.

It is suggested that a more complete and useful surveillance of salmonellosis for large urban areas can be attained by analysis monthly of municipal primary and secondary waste treatment plant sludges.

Results obtained can indicate current high carrier rates of certain Salmonella serotypes or suggest the presence of pathogens in local food or feed products that may need inspection to avert spread.

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SECTION I

CONCLUSIONS

1. The rate of die-off of the pathogens, Salmonella species and Pseudomonas aeruginosa, even in secondary treatment effluents is considerably slower than that of the "indicators of pollution" i.e., fecal coliforms and fecal streptococci. Chlorinated primary effluents and package plant effluents contain considerable numbers of pathogens.
2. The use of untreated primary and biological treatment (secondary) sludges for application to agricultural grass lands shows persistence of the pathogens, Salmonella sp. and Ps. aeruginosa for periods extending to 21 weeks.
3. Analysis of municipal treatment sludges for Salmonella sp. indicate high carrier rates in urban populations.
4. A sensitive quantitative method for the simultaneous detection and enumeration of Salmonella species and Pseudomonas aeruginosa from wastewaters, treatment effluents, treatment sludges, receiving waters, finished waters, and sludge-treated soils is presented.

SECTION II

RECOMMENDATIONS

1. Chlorination of primary treatment plant effluents is evidently not sufficient to obtain the desired elimination of indicator bacteria to meet requirements for receiving waters. Therefore, further treatment is necessary to eliminate the indicator bacteria, as well as the pathogens. Package treatment plant (extended aeration) effluents are generally of low quality, especially when it is considered that often the only receiving waters are those of the plant output. Further studies of disposal should be made.

The methods described can be useful for monitoring drinking water supplies that are insufficiently chlorinated or not treated at all.

2. Municipal treatment plant sludges should not be applied to crop or grass lands unless some means of disinfection are employed at the treatment plant before distribution.
3. It is suggested that a more complete and useful surveillance of salmonellosis in large urban areas can be attained by analysis of municipal waste treatment sludges at regular intervals.

SIMULTANEOUS QUANTITATION OF SALMONELLA SPECIES AND
PSEUDOMONAS AERUGINOSA. I. POLLUTED WATERS

INTRODUCTION

For effective determination of the presence of disease-producing bacteria in polluted waters and wastewater treatment effluents, quantitative methods are needed (6, 7, 9, 10, 32, 33) for detection of *Salmonella* and other pathogens.

The coliforms, fecal coliforms, and the fecal streptococci are some of the "indicators of pollution" from human and animal fecal sources. These indicators, however, do not indicate how dangerous the pollution might be.

Data obtained by purely qualitative methods for salmonellae, such as that obtained by the gauze pad method (26), wherein the water from sewer outfalls or surface waters pass through the pad for several days, cannot be validly compared with values obtained for total or fecal coliforms per 100 ml of water samples at the same location to obtain ratios of coliform or fecal coliform to Salmonella species. Actual numbers of Salmonella sp. per 100 ml can be validly compared with total or fecal coliform counts per 100 ml.

Data accumulated by the method herein described does not warrant blanket statements as to the ratio of indicator bacteria to pathogens. Indeed, one reference (24) states that water supplies containing many Ps. aeruginosa and few or no coli-aerogenes were found to be relatively common in Northern Israel. In the recent (1965) Salmonella typhimurium epidemic in Riverside, California, the pathogens were present in the drinking water supply, allegedly in the absence of the indicator bacteria (4).

Morgan, et al (21) and Bonde (1) have recently criticized the employment of membrane filter techniques when testing a variety of waters, and find the multiple tube techniques (MPN) to be more consistent and applicable over a wider range of conditions. The author finds that MF methods produce more false positives on transfer to secondary medium than do multiple tube methods when employed for pathogen detection.

Research on practical methods for the quantitation of pathogenic bacteria which are important to health and water quality is an approved project of the National Environmental Research Center, Cincinnati.

MATERIALS AND METHODS

Multiple Tube Method

Four factors employed produced a simple and effective multiple tube method for the quantitation of Salmonella sp. and Pseudo-monas aeruginosa:

1. Enrichment medium Dulcitol selenite broth (DSE) Raj (22) modified, enhanced by growth factors furnished by the addition of 10% by volume, of an 18-hour 37 C culture of S. paratyphi A in 1xDSE, that has been killed by heating to 90 C. See Table 1 for medium formula.

Table 1. Composition of DSE Medium 1x (Modified) Raj (22)

Proteose peptone (Bacto)*	0.4%
Dulcitol	0.4%
Sodium <u>acid</u> selenite (BBL)**	0.5%
Na ₂ HPO ₄	0.125%
KH ₂ PO ₄	0.125%
Distilled water	q.s.

Mix and dissolve constituents thoroughly in sterile flask, bring temperature to 88 C in water bath to obtain clear medium, distribute, as desired, in sterile screw-cap tubes in amounts for multiple tube tests, as illustrated in Table 2. Adjustment of pH is not necessary if acid sodium selenite is employed.

* Difco Laboratories, Inc., Detroit, Michigan 48201, USA

** Division of BioQuest, P. O. Box 175, Cockeysville, Maryland 21030, USA

2. Incubation at $40\text{ C} \pm 0.2$ is critical for primary enrichment. Livingstone (14) recommended 40 C for secondary plating. See Table 6.
3. The swimming ability of *Salmonella* sp. (and *Ps. aeruginosa*) as cited by Livingstone (14), Ino and Graber (8), and Stuart and Pivnick (27), enables the pathogens to migrate to the surface area of the enrichment broth after the incubation period of one and two days. The surface "scum" is picked up using a 7-mm (22 gauge) nichrome or platinum loop and streaked for isolated colonies onto the secondary agar medium plates. To obviate MPN "skips" in the first row of 2x DSE broth in which 10-ml sample has been added per tube, a surface loopful is transferred to each of two sections of a divided plate of XLD agar and streaked for isolated colonies.
4. Xylose lysine desoxycholate agar (XLD) Taylor (28) as the secondary plating medium enables one to obtain typical black-centered *salmonella* colonies, and flat, erose, grayish, slimy *Ps. aeruginosa* colonies on a pink or red background from enrichment cultures streaked at 1 and 2-day intervals.

Other considerations employed for successful testing is the use of Reeve-Angel 984 H ultra glass fiber filters (47 mm) to concentrate the bacteria which may be present in larger volumes of clear waters, using 1-gallon or 10-gallon samples. The schemata for five methods is illustrated in Table 2.

Salmonella Detection

The streaked XLD agar plates are incubated, in inverted position, at 37 C or preferably at 40 C for a period not to exceed 24 hours. Black-centered and pink non-mucoid (rare) colonies are picked to triple sugar iron agar (TSIA) slants, or Kligler iron agar (KIA) slants that are streaked and the butt stabbed. Typically, the butt of the tube is acid with blackening hydrogen sulfide, and the slant appears either alkaline red or does not change color after 18 - 24-hour incubation at 37 C or 40 C. In more than 90% of our picks, the TSIA or KIA slant cultures were urease negative after purification of initial cultures on XLD plates, and picking black-centered translucent-edged colonies or pink opaque (non-mucoid) colonies to TSIA slants.

Pseudomonas aeruginosa Detection

The XLD agar plates described above are also examined for flat, erose, grayish-pink, slimy colonies that are picked to King A

agar (12) slants (available as Tech agar [BBL]) streaking and stabbing the butt (in the absence of the flat, slimy colonies, any slimy colony on a pink background may be picked as there may be a mixture with other pseudomonads). These tubes are labelled according to their MPN position and incubated at 40 C. The blue-green pigment, pyocyanin, that is produced only by *Ps. aeruginosa*, is often apparent in slant cultures after 6 hours at 40 C. After 18 - 24 hours incubation, the blue pigment will be apparent in positive cultures, and a reddish pigment (pyorubin) may start to develop. The blue pyocyanin can be extracted from the positive slant cultures by the addition of chloroform.

Table 2. Suggested Schemata for Testing Water Samples for *Salmonella* sp. and *Ps. aeruginosa*

A. Direct MPN (turbid samples) 5-tube 5-dil MPN/100 ml										B. 5-tube 5-dil MPN/liter (1,000 ml)									
Media employed		Sample amount	A	B	C	D	E			Media employed		Sample amount	A	B	C	D	E		
10 ml 2x DSE	10 ml	10 ml	10	10	10	10	10	direct	mix	20 ml 1x DSE	100 ml	100 ml	Pad*	Pad*	Pad*	Pad*	Pad*	mix	
9 ml 1x DSE	1 ml	1 ml	1	1	1	1	1	direct	mix	9 ml 1x DSE	10 ml	2 ml	2 ml	2 ml	2 ml	2 ml	2 ml	of above pad-dil.	mix
9 ml 1x DSE	0.1 ml	1 ml	1 ml	1 ml	1 ml	1 ml	1 ml	1 ml	of above dil.	mix	9 ml 1x DSE	1 ml	1 ml	1 ml	1 ml	1 ml	1 ml	of above dilution	mix
9 ml 1x DSE	0.01 ml	1 ml	1 ml	1 ml	1 ml	1 ml	1 ml	1 ml	of above dil.	mix	9 ml 1x DSE	0.1 ml	1 ml	1 ml	1 ml	1 ml	1 ml	of above dilution	mix
9 ml 1x DSE	0.001 ml	1 ml	1 ml	1 ml	1 ml	1 ml	1 ml	1 ml	of above dil.	mix	9 ml 1x DSE	0.01 ml	1 ml	1 ml	1 ml	1 ml	1 ml	of above dilution	mix
Read MPN table for "+" tubes directly for numbers/100 ml										Read MPN table for "+" tubes - reduce result by one decimal place for numbers/100 ml									
C. One-Gallon MPN Test (moderately clear samples) MPN/Gal.										D. 5-tube 5-dil MPN/10 liters (10,000 ml)									
Media employed		Sample amount	A	B	C	D	E			Media employed		Sample amount	A	B	C	D	E		
20 ml 1x DSE	380 ml	380 ml	Pad*	Pad*	Pad*	Pad*	Pad*	mix		20 ml 1x DSE	1000 ml	1000 ml	Pad*	Pad*	Pad*	Pad*	Pad*	mix	
9 ml 1x DSE	38 ml	2 ml	2 ml	2 ml	2 ml	2 ml	2 ml	2 ml	of above pad-dil.	mix	9 ml 1x DSE	100 ml	2 ml	2 ml	2 ml	2 ml	2 ml	of above pad-dil.	mix
9 ml 1x DSE	3.8 ml	1 ml	1 ml	1 ml	1 ml	1 ml	1 ml	1 ml	of above dil.	mix	9 ml 1x DSE	10 ml	1 ml	1 ml	1 ml	1 ml	1 ml	of above dil.	mix
9 ml 1x DSE	0.38 ml	1 ml	1 ml	1 ml	1 ml	1 ml	1 ml	1 ml	of above dil.	mix	9 ml 1x DSE	1 ml	1 ml	1 ml	1 ml	1 ml	1 ml	of above dil.	mix
9 ml 1x DSE	0.038 ml	1 ml	1 ml	1 ml	1 ml	1 ml	1 ml	1 ml	of above dil.	mix	9 ml 1x DSE	0.1 ml	1 ml	1 ml	1 ml	1 ml	1 ml	of above dil.	mix
Read MPN table directly for numbers/gal. Divide result by 3.785 for numbers/100 ml										Read MPN table for "+" tubes - reduce result by two decimal places for numbers/100 ml									
E. Ten-Gallon MPN Test (potable waters) MPN/10 Gal.																			
As above, except 3800 ml filtered* for each tube of first row of tubes. Read MPN table directly for numbers/10 gal. Divide results by 37.85 for No./100 ml																			
* Indicated amount of sample filtered through Reeve-Angel 984M ultra glass fiber filters 47 mm, sterilized, rough side up in glass Petri dishes. (Autoclave 115.2 C/10 min). Available directly from H. Reeve Angel and Company, 9 Bridewell Place, Clifton, N. J. 07014. After sample filtration, double pliable filter over with sterile, alcohol-flamed forceps, and insert into appropriate first row tube of MPN series. Shake tube vigorously. Occasionally more than one filter may be required to filter the entire amount for each tube.																			

Precautions and Other Information

If the TSIA and King A agar slants are in screw-cap tubes, the caps must be loosened prior to incubation at 40 C in order to obtain desired reaction. Smith and Anderson-Langmuir (25) have described variable results when using tightly closed screw-cap tubes. Caps of the multiple-tube series should also be loosened before incubation.

Information regarding sampling, preparation of XLD agar plates, urease testing, serological identification, and safety precautions is presented in the Appendix.

RESULTS

Late in this study we found that none of the data presented in Table 3 indicated isolation of S. paratyphi A. A dilution of an 18-hour TSIA slant culture of S. paratyphi A was added to a trickling filter effluent so that approximately 10 - 20 of these pathogens would be present per 100 ml. The MPN value for the seeded sample, counting only the S. paratyphi A, was 4-0-0 or 13/100 ml. The salmonella count for other serotypes in the seeded series was 5-3-2 in the 5-tube series or 140 Salmonella/100 ml, which included S. cubana (13 strains), S. tennessee (1 strain), and Arizona (1 strain). A control series without seeding was run at the same time with a MPN value of 4-2-0 in the 5-tube series or 22 Salmonella/100 ml. Apparently, the addition of the seed culture enhanced the growth and isolation of other serotypes. Subsequent tests employing 10% by volume of a killed culture in DSE medium showed invariably higher MPN values and isolated black-centered colonies than control series in DSE without enhancement.

The method described above employing DSE (without enhancement) resulted in the first successful routine quantitative isolation of Salmonella sp. from less than 100 ml of municipal sewage. Subsequent tests of other polluted waters such as rivers, storm-water runoff, package plant effluents, chlorinated primary treatment effluents, secondary activated sludge treatment effluents, trickling filter effluents, etc., showed pathogens to be surprisingly ubiquitous and present even in waters with relatively low "indicator" counts.

In Table 3, the majority of identified Salmonella serotypes appear in the sewage (MS), sludges, and treatment plant effluent columns mainly because more samples were tested in these categories. Sample of streams and stormwater runoff were fewer and hence

results were not as conclusive. However, since employing the "per gallon" technique the later types of samples invariably contain Salmonella species, Ps. aeruginosa, and occasionally Shigella. Recent studies employing the "per liter" "per gallon," "per 10 liter" and the "per ten gallon" techniques show that Salmonella sp. and/or Ps. aeruginosa can be isolated routinely

Table 3. Distribution of Serotypes of Salmonella in Wastewaters

No.	Serotype	Group	MS ¹	Sludges ²			Treatment Effluents ³				Str. ⁴	SMR ⁵	Isolated No. of Strains	Rank Human Occur. %
				PS	ADS	SAS	PE	SE	PPE	TFE				
1	albany	C2		x									17	
2	anatum	E1	x	x	x	x		x			x		127	13
3	bareilly	C1	x									x	42	23
4	benfica	E1	x										5	
5	binosa	E2				x		x					29	24
6	blockley	C2		x	x	x	x	x		x			93	11
7	braenderup	C1		x	x	x							13	
8	brancaster	B				x							1	
9	bredeney	B		x									8	23
10	california	B				x							2	
11	chester	B	x	x	x	x	x	x					203	19
12	cholera-suis	C1	x	x									30	
13	" var. kunsendorf	C1	x	x									29	
14	cubana	G	x	x	x	x		x	x				132	20
15	derby	B	x	x	x	x	x						284	12
16	drypool	E2				x							10	
17	enteritidis	D	x	x	x	x	x	x			x		113	5
18	friedensau	G				x							4	
19	give	E1	x	x	x	x		x					20	22
20	haifa	B				x							2	
21	havana	G	x	x		x							16	
22	heidelberg	B	x	x	x	x		x			x		110	6
23	indiana	B	x										10	
24	infantis	C1	x	x	x	x	x	x			x		96	9
25	java ^b	B	x	x		x		x				x	15	7
26	javiana	D	x	x	x	x	x	x					13	8
27	kottbus	C2	x	x	x	x		x			x		158	
28	lexington	E1				x	x						33	
29	litchfield	C2				x		x					17	21
30	livingstone	C1	x	x		x	x						52	
31	lomita	C1			x			x			x		26	
32	manhattan	C2	x	x	x	x							73	14
33	meleagridis	E1					x				x		18	25
34	mission	C1		x									14	
35	montevideo	C1	x	x		x							47	16
36	muenchen	C2	x	x	x	x					x	x	45	18
37	newington	E2	x										2	
38	newlands	E1				x							8	
39	newport	C2	x	x	x	x	x	x	x		x	x	161	4
40	norwich	C1		x	x	x		x			x		14	
41	ohio	C1									x		19	
42	oranienberg	C1				x		x					44	15
43	paratyphi B	B	x	x	x	x		x	x				35	17
44	preston	B		x		x							2	
45	reading	B	x	x	x	x	x	x		x			26	
46	rubislaw	F		x		x							15	
Sub total													2,233	

from streams, stormwater runoff, chlorinated primary effluents, secondary activated sludge and trickling filter effluents and some lime-carbon column secondary chemical treatment effluents. Any water source from which the nutrients have not been adequately removed and properly chlorinated may be suspect, and should be tested by the 10-liter or the 10-gallon method.

(Continued)

No.	Serotype	Group	MS ¹	Sludges ²			Treatment Effluent ³				Str. ⁴	SWR ⁵	Isolated No. of Strains	Rank Human Occur.**
				PS	ADS	SAS	PS	SE	PPE	TFE				
47	saint paul	B				x		x					19	10
48	san diego	B	x	x	x	x		x		x			44	18
49	schleissheim	B	x	x		x							12	
50	schwarzengrund	B	x			x							37	
51	seeftenberg	B ₄		x	x	x							30	25
52	simsbury	B ₄		x	x	x							7	
53	taksony	B ₄					x						16	
54	tennessee	C ₁						x					12	20
55	thompson	C ₁	x	x		x		x		x		x	63	3
56	tahiongue	C ₂	x	x		x		x					41	
57	typhimurium*	B	x	x	x	x	x	x	x	x	x	x	288*	1
58	" var. copenhagen*	B	x										•	6
59	typhi-suis var. veldagense	C ₁		x									2	
60	usaramo	H	x					x					28	
61	wil	C ₁		x	x								3	
62	worthington	G	x		x				x				10	24
Incomplete serology ***			x	x		x		x	x	x			193	
Arizona Group			x	x		x			x	x	x	x	111	
Total													3,149	

b Bioserotype.

* After first 9 strains of var. copenhagen identified. No further effort made to differentiate between serotype typhimurium and its variety.

** According to Martin and Bring, Table 1, Appl. Microbiol. 17:111-117 (1969).

*** Partial "O" and "H" antigens obtained; insufficient to name serotype.

MS¹: Municipal and Industrial Sewage.

Sludges²: PS, Primary sludge; ADS, Anaerobic digested sludge; SAS, Secondary activated sludge.

Treatment Effluents³: PE, Primary effluent; SE, Secondary effluent; PPE, Package plant effluent; TFE, Trickling filter effluent

Str.⁴: Rivers and other stream waters

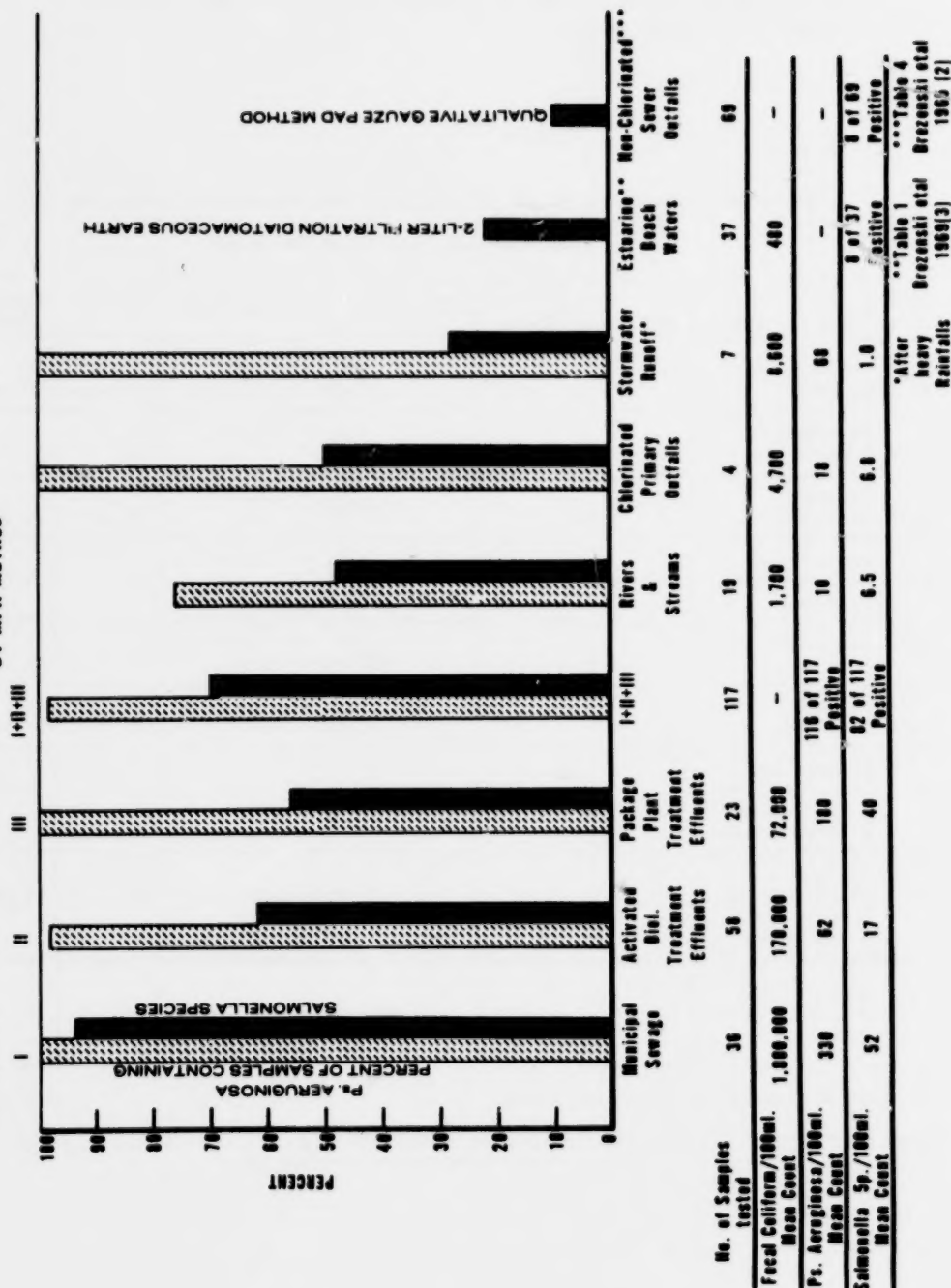
SWR⁵: Stormwater runoffs

Table 3 represents a summation of Salmonella serotypes isolated and identified over a period of one and half years by this laboratory mainly from samples collected in the Cincinnati area. It should be noted that 24 of the first 25 serotypes of Salmonellae ranked according to human occurrence by Martin and Ewing (17) were isolated.

In Table 4, the percent of Ps. aeruginosa and Salmonella sp. isolations for the various types of samples are illustrated graphically, and the mean fecal coliform (M-FC) and mean pathogen counts are given for 147 samples tested by the multiple tube method at 40 C. It should be pointed out that over 90% of the results were obtained from less than 100 ml of water samples. For comparison, the last two salmonella bars on the graph represent the results obtained by Brezinski, et al., 1969 (3), and 1965 (2), using 2-liter samples filtered through diatomaceous earth, and the gauze pad method, respectively. The results obtained from testing sewage influents and the effluents from extended aeration plants are interesting because of the growing popularity of package treatment plants in suburban housing development areas. Small receiving streams usually do not dilute package plant effluents to any extent. During warm, dry periods the treatment effluents are often the only water in small stream beds and produce foul odors and other conditions which have ruined the aesthetic appeal of many areas. The package plant results also illustrate that although the fecal coliforms in the effluents are reduced 96 percent, the mean numbers of Ps. aeruginosa (as compared to sewage) are only reduced 40 - 50 percent, and the mean salmonella numbers are only reduced 7 - 10 percent. In one sampling of a small capacity package plant effluent the Ps. aeruginosa count (16,000/100 ml) was four times that of the fecal coliforms (4,000/100 ml).

Table 5 illustrates the value of employing the fiber glass ultra filters to detect salmonella in relatively clear filterable water samples. Studies of the bacterial retention of ultra filters of the 984 H-type showed them to retain greater than 98 percent of an aqueous dilution of a stock culture of E. coli. The first example of salmonella yield per 100 ml gave a value of 4.5/100 ml (equivalent to 170/gal), the per gallon yield gave a MPN value of 210 Salmonella sp. In the next example, no salmonella were isolated by the per 100 ml method, and the "per gallon" yield was 7.3, etc. More than five times as many salmonella were picked from the per gallon MPN tubes, with the additional advantage of isolating as high as five times as many serotypes. That the filtration technique is a valuable adjunct to the detection and enumeration of Salmonella sp. is self-evident throughout Table 5, as is the fact that the per 10 liter

TABLE 4.
PERCENTAGE OF PATHOGEN ISOLATES AT 40C FROM VARIOUS TYPES OF WASTEWATERS AND TREATMENT EFFLUENTS
BY MPN METHOD



or per 10-gallon technique should be valuable for testing unchlorinated drinking water sources to insure the absence of pathogenic bacteria.

Table 5. Advantages of the Ultra-Filter MPN Method for Detecting Salmonella in Filterable Samples by the Per Gallon or the Per Ten-Gallon Technique

1970 Date	Type of Sample	MPN Count/100 ml	Number/gal = equiv./100 ml 37.85	Strains /100 ml	Strains /gal
8/ 3	Stormwater runoff	4.5	$\frac{210}{37.85} = 5.55$	7 (1)*	21 (2)*
8/10	Stormwater runoff	< 3	$\frac{7.3}{37.85} = 0.193$	0	6 (2)*
8/17	Sewage municipal	6.2	$\frac{1,500}{37.85} = 39.63$	3 (1)*	19 (5)*
8/17	Secondary act. sludge effluent	< 3	$\frac{3.6}{37.85} = 0.095$	0	4 (1)*
8/24	As above	< 3	$\frac{28}{37.85} = 0.74$	0	10 (2)*
8/24	Sewage municipal	< 3	$\frac{110}{37.85} = 2.91$	0	17 (4)*
9/ 1	Mississippi River ml. 403.6 (4 day old iced sample)	3	$\frac{93}{37.85} = 1.93$	2 (1)*	5 (1)*
9/ 1	As above River ml. 403.1 (4 day old iced sample)	43	$\frac{11,000}{37.85} = 290.6$	10 (1)*	35 (3)*
10/12	Rural well Clermont County Ohio	-	**< 1.6 per 10 gallon		-

* Number of Salmonella serotypes present

** Ps. aeruginosa 11/10-gallon (equiv. 0.026/100 ml)
Fecal coliforms 1/100 ml, Fecal streptococci 84/100 ml
Total aerobic count 22,000/100 ml

Table 6 illustrates graphically the comparative advantage of using 40 C, instead of 37 C or 41.5 C for the enrichment procedure. The new 1971, 13th Edition of Standard Methods (APHA) states that either 37 C or 41.5 C can be used as a matter of choice for the isolation of Salmonellae.

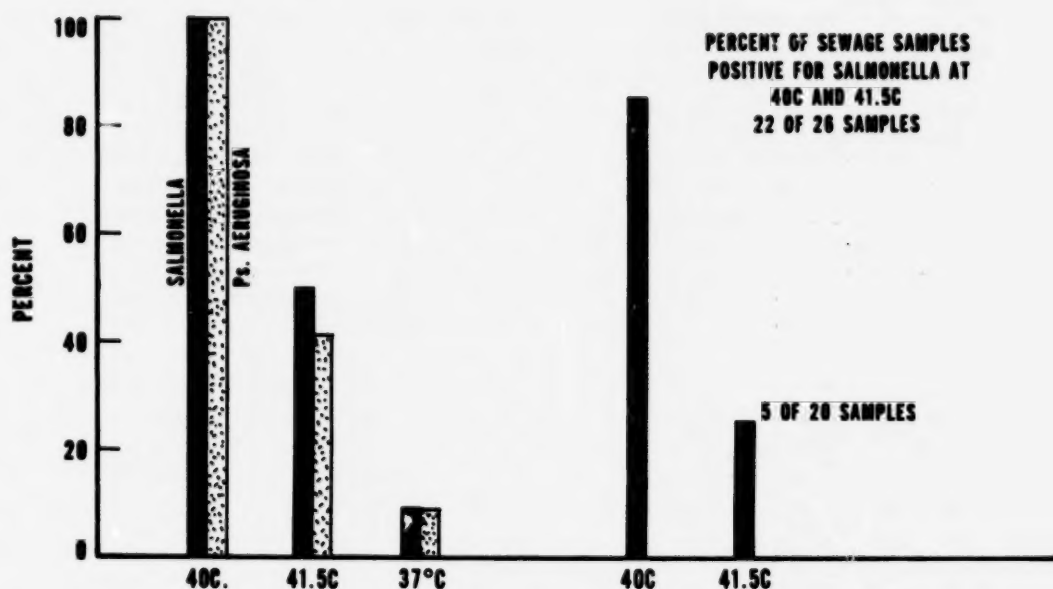


TABLE 6.
RELATIVE NUMBERS OF PATHOGENS ISOLATED FROM IDENTICAL SEWAGE SAMPLES IN DSE
ENRICHMENT BROTH AT 3 DIFFERENT TEMPERATURES

DISCUSSION

The general view that Salmonellosis is a food-borne infection, and that human infections require large numbers of salmonella to produce food poisoning is stated by McCullough and Eisele (18). However, the virulence of salmonella is increased by passage through humans and animals, thus requiring fewer numbers to become infectious. It is possible that contact sports in polluted waters could be a source of infection via the nasal passages or conjunctival route. In guinea pigs, infection with minute doses of Salmonella enteritidis and S. typhimurium by the conjunctive route set up conjunctivitis followed by a blood-borne infection via the cervical lymph nodes (20). These infections occurred under natural as well as experimental conditions. The spleen and the gut also became infected in a majority of cases during a rapidly spreading epizootic of salmonella infection. That this route could be a source of human infection cannot be ruled out as evidenced by a case report (15). A mixed culture of 25 S. sofia and S. bovis-morbificans introduced into the left antrum of a human volunteer produced acute bilateral sinusitis within four hours after administration, and vomiting and diarrhea 37-1/2 hours thereafter. "The organisms were recovered from the purulent sinus discharge, sputum, and feces." Treatment with long-acting sulphonamides failed with relapse after seven days. Recovery finally was obtained with chloramphenicol therapy. (15).

Waters that animals come in contact with, or pollute with urine or feces, could be a source of zoonoses. A report of zoonoses in the South (U.S.A.) by Martin (16) shows the occurrence of salmonellosis and six other bacterial diseases in all the seventeen reporting States and the District of Columbia.

S. typhi was not isolated by the method used in this study. Moore (19) cites the work of E. O. Jordan (1895) who in a classic study immersed typhoid suspensions in semipermeable sacs of parchment or colloidion in the waters of Lake Michigan and the heavily polluted Chicago Drainage Canal. In the relatively clean waters of the lake, most died out in 4 - 7 days; in the polluted waters, no typhoid bacilli were recovered from the sacs after two days.

Previous efforts by the author (11) to devise methods for the quantitation of pathogens in wastewaters have been subject to many errors which prevented a true quantitation of the desired microorganisms. The most common of these errors were the use in enrichment broths of compounds of varying chemical composition and, therefore, of varying selectivity for the pathogens or the

use of incubation temperatures in the enrichment procedure which were too low (37 C) or too high (41.5 C). The employment of stock cultures to test out new methods for the isolation of pathogens is a waste of effort. Instead, water sources for which the method is being devised should be used. Secondary plating medium, which do not reduce the numbers of viable pathogens, or allow excessive growth of the more predominant false-positive organisms should be used (11, 23, 29). Salmonellae are usually a definite minority of the Enterobacteriaceae present in polluted water samples. The probability of distinctive colonial isolation on membrane filter culture after enrichment are much less than in multiple tube methods wherein the enrichment culture can be spread over a larger surface on a selective secondary medium for the appearance of isolated (distinctive) colonies.

The antibiotic resistance of Ps. aeruginosa, and the ubiquity of this human pathogen in all samples of wastewaters and treatment effluents thus far studied is distressing. Even tap water drinking supplies are not immune to this increasingly important bacteria. Ps. aeruginosa have been isolated several times from faucets with aerators, and from refrigerated water drinking fountains prior to more recent high chlorine residuals in municipal water supplies. The role of Ps. aeruginosa in human infection has become increasingly important, and is the subject of many reports too numerous to cite. All of the current procedures for the isolation of Ps. aeruginosa depend upon the production of pyocyanin, but give equivocal results, at the low incubation temperature of 37 C. Masking of pigment production occurs whether membrane filter or multiple tube methods are used.

Although a moderately successful combination for membrane filter isolation of the salmonella was found, it soon became obvious, when comparing the best of the MF combinations with multiple tube tests employing DSE and GN enrichment broths tests and XLD secondary plating agar, that the latter methods were best. The GN-XLD combination gave slightly fewer isolations than did the DSE-XLD method. Use of the DSE-XLD method consistently allowed the isolation of Salmonella from municipal sewage, and when employing the factors already discussed it was found that Salmonella species and Ps. aeruginosa were surprisingly ubiquitous during all seasons of the year regardless of the source of wastewater or treatment effluent.

Secondary agar plating media that were found to be either non-productive in 24 hours or to allow the growth of too many "false positive" enterobacteriaceae were brilliant green agar (BGA).

Salmonella-Shigella agar (SS), bismuth sulfite agar (BSA) (freshly prepared or aged in refrigerator), and Hektoen Enteric agar (HE) (13). In a comparative study, Hektoen Enteric agar yielded more black-centered colonies than XLD from the same MPN series; however, 77% were false positive (urease) and only 23% were Salmonella species. The MPN count for Salmonella sp. from the DSE-HE combination was half that employing the DSE-XLD combination. The HE agar was not useful for detecting Ps. aeruginosa.

APPENDIX

Collection and Testing of Samples

Samples are collected in sterile glass or polypropylene containers. If the presence of chlorine is known or suspected, one ml of a sterile aqueous 10% sodium thiosulfate solution is added. The pathogens do not seem especially sensitive to refrigeration and have been detected in high numbers in iced river water samples after periods as long as four days. Testing samples within a 24-hour period is desirable. Sludge samples with relatively high salmonella counts yielded 62% of the original numbers after five weeks storage in the refrigerator, and after ten weeks 10% of the original count. Of course, if indicator counts are desired refrigerated samples must be tested within 24 hours. Some of the samples reported were received by airmail, special delivery, at ambient temperature and still contained high counts of both pathogens.

Preparation of XLD Agar

The XLD agar utilized in this procedure was devised by Taylor (28). Throughout the tests we have employed commercially dehydrated XLD agar from BBL (a Division of BioQuest, P. O. Box 175, Cockeysville, Maryland, 21030, USA) and Difco (Difco Laboratories, 920 Henry Street, Detroit, Michigan, 48201, USA) and found it to be non-toxic and equivalent to the XL agar base with added selective system preferred by Taylor and Schelhart (30) if certain precautions were taken in its preparation. Each new lot of commercial medium must be tested for the appropriate pH, and if lower than pH 7.3 sufficient 1N NaOH added to each rehydrated lot to bring the pH to 7.4. The BBL XLD agar rehydrated in a sterile flask was heated directly with frequent swirling to a temperature not greater than 88 C, and in the same manner Difco XLD agar rehydrated was heated to a temperature not greater than 92 C, and cooled in a water bath to 52 - 55 C before distribution to sterile 15 x 100 mm petri dishes. The dishes are allowed to cool with tilted tops at room temperature for about an hour and then inverted overnight at room temperature before use, or placed in sealed, plastic sleeves in the refrigerator until used.

Urease Testing

A micro-test for urease testing of TSIA slant cultures that the author has found to be useful utilizes an autoclave sterilized medium devised by Tidwell, et al. (31). For use, 0.5 ml portions of the sterile broth medium are pipetted into sterile 10 x 75 serological tubes, and inoculated with 3 mm loopfuls of culture, and

incubated at 37 C. The tubes may be closed with sterile-size 0 corks. Urease positive cultures (red) are usually evident within a 4-6 hour period and are discarded. As the salmonella are urease-negative only purified cultures that do not change the medium to a dark-red in 4-6 hours are kept for serological identification.

The medium formula is yeast extract 0.01 gm, KH_2PO_4 0.91 gm, $\text{NaH}_2\text{PO}_4\text{-H}_2\text{O}$ 0.95 gm, urea 2.0 gm, phenol red (ws) 0.0011 gm, distilled water 97.7 ml. The medium has a pH of 4.8 prior to autoclaving at 121 C for 15 minutes. The final pH is 6.8. May be stored in screw-cap bottles in refrigerator until used.

Serological Identification of Salmonella

Typical purified slant cultures on TSIA can be identified as Salmonella species serologically according to the needs of the laboratory performing the tests. After initial negative urease tests have been obtained, slide agglutination tests may be performed employing Bacto-Salmonella "O" antiserum poly A-I, or "H" broth tube tests may be performed using Bacto-Salmonella "H" antiserum Poly a-z to detect flagellar antigens of the Salmonella species. These tests and the methods for complete identification of Salmonella serotypes are described by Edwards and Ewing (5), and by Difco Technical Information 0168 (April 1968).

Use of the Most Probable Number (MPN) Tables

When appropriately marked cultures from 10A, 10B, 10C, etc. tubes of the MPN series are positive by the Salmonella antisera tests, a + is placed in the MPN column, and using the usual MPN (most probable number) table, the number per 100 ml, per liter, per gallon, per 10 liters, or per 10 gallon can be read directly, i.e., a schema giving positive readings of 5 + (10 ml), 3 + (1 ml), 1 + (0.1 ml) tubes would indicate 110 salmonella /100 ml; or 5 + (380 ml), 3 + (38 ml) 1 + (3.8 ml) tubes would indicate a count of 110 salmonella/gal., similarly a series commencing with 5 + (3800 ml) etc. would give a count per 10 gallon.

Safety Precautions

Throughout our studies of pathogens our laboratory personnel have used a 1 - 500 solution of Hexadecyltrimethylammonium bromide (CTAB) for use on hands before and after handling contaminated material, and for washing lab bench tops. The solution costs less than 25 cents per gallon (CTAB 7.6 gm per gallon of ~~tap~~ water).

de-ionized water or distilled

Technicians handling these materials should not rub the ears and eyes.

II. PERSISTENCE OF PATHOGENS IN SLUDGE TREATED SOILS

INTRODUCTION

The results of this study show that the use of unsterile municipal waste treatment sludges on crop or grass land to obtain the beneficial effects of water, nutrients, organic matter, trace metals, etc. may well contribute to uninterrupted human and animal disease cycles.

Much has been written concerning land "disposal" of unsterile municipal treatment sludges by frequent application to crop or even public park land (1, 5, 7, 8). Many have shown concern about the ill effects of directly applying anaerobically digested sludges to soil which results in inhibition of seed germination, odor problems, and concentrations of zinc, manganese, chromium, copper, lead and nickel which are too high. However, little has been published about persistence of pathogenic bacteria in treated soils for long periods of time. The bacterial picture is entirely dismissed from consideration in nearly every report.

This paper will consider the bacteriological results of single applications of unsterile municipal sludges on crop and grass land. Various types of sludges were used in this study. The persistence of Salmonella species and Pseudomonas aeruginosa were determined by a quantitative method devised by Kenner (6), and of E. coli (fecal coliform) by a membrane filter method.

Although application of certain types of sludges do give beneficial results to crop and grass land, it is evident from the results obtained that due consideration should be given to where and when these sludges can be applied safely.

INITIAL PENNSYLVANIA STUDIES

Initial studies regarding the persistence of pathogens in the soil of sludge-treated farm land were obtained from treated soil samples received at weekly intervals from St. Marys and Penn Township (Hanover) Penn., via airmail, special delivery. J. O. Evans designed the studies, and Dotson and Evans (3) reported them. Detailed description of study plan and sampled areas is reported by Cooke (2) who employed the same samples for the study of fungi in sludge-treated soil samples. These studies took place during the relatively warm period from April 2, 1968 through August 27, 1968. The agricultural sites selected for the application of sludges at St. Marys and Penn Township were as nearly level as possible, and the soil samples from treated sites did not contain fecal coliforms, Ps. aeruginosa, or Salmonella sp. prior to application.

The St. Marys site received an application of anaerobic digested, activated sludge of 1-1/2 gallons per sq. ft (5.68 liter per 30.48 cm²), activated sludge plant 1.3 MGD (4.9 MLD) typically 5% solids. Bacteriological assay of the sludge showed that 1.8×10^7 fecal coliforms, 56,000 Ps. aeruginosa, and 414 Salmonella sp. were applied per sq. ft (30.48 cm²) of land. After application, two soil samples were obtained by pressing sterile ointment tins into the soil site, and mailed special delivery, airmail to our laboratory for initial assay. The initial soils contained 2400 fecal coliforms per gram, 10 Ps. aeruginosa per gram, and Salmonella sp. were not detected initially or throughout the study. At the end of 21 weeks fecal coliforms were still detectable (192 per gram); Ps. aeruginosa was only detectable initially.

The Penn Township site received an application of aerobic digested, activated sludge of one gallon per sq. ft (3.785 liters per 30.48 cm²). Bacteriological assay of the sludge showed that 2.5×10^7 fecal coliforms, 3.78×10^6 Ps. aeruginosa, and 5,678 Salmonella sp. were applied per sq. ft (30.48 cm²) of land. Initial assay of soil samples after application showed them to contain 550 fecal coliforms per gram, 100 Ps. aeruginosa per gram, and 0.36 Salmonella sp. per gram. Weekly samples (2) received thereafter showed that at 18 weeks 22 fecal coliforms per gram persisted, while Ps. aeruginosa persisted for 16 weeks, and Salmonella sp. were last detected at 10 weeks. The anaerobic sludge contained only one serotype of Salmonella, namely S. infantis. The aerobic sludge contained S. infantis, S. litchfield, and S. derby. The final type detected in the soil at 10 weeks was S. derby.

As the initial studies were conducted only through a relatively mild spring and a hot summer, it was thought desirable to study the effect of late fall and winter months' weather on the persistence of pathogens in a location where sampling of the soils and bacterial assays could be affected within a few hours of sampling.

Studies made of indicator and pathogen persistence in the period between November 6, 1969 to June 8, 1970:

Site

A level, grassy plot of ground, east of the Research Center, was chosen by the soil scientist. Three measured areas, each 2 x 5 feet, (0.610 x 1.524 meters) were marked off, and each was enclosed by a corrugated plastic strip fence driven into the ground so that the strip protruded approximately 1/2 inch (1.25 cm) above ground level. The fence prevented runoff from the other areas during heavy rainfall.

PRELIMINARY SOIL SAMPLES OF MARKED OFF AREAS

Soil samples from the three plot areas were taken November 5, 1969 and were devoid of fecal coliforms, Ps. aeruginosa, and Salmonella sp. Soil samples in the vicinity, and directly outside the plots were negative when checked April 6, 1970. Mowing of areas commenced in May 1970.

ANALYSIS OF SLUDGES APPLIED TO THE THREE PLOTS

Plot 1: Anaerobically digested, primary sludge applied at rate of 1 gallon/sq. ft (3.785 liters/30.48 cm²) on 11/5/69. (Source, Millcreek Treatment Plant - Cincinnati, Ohio).

Fecal coliforms per gallon 1.48 x 10⁷ (390,000/100 ml)

Ps. aeruginosa per gallon 1,300 (34/100 ml)

Salmonella sp. per gallon 1,098 (29/100 ml)

Plot 2: Undigested activated sludge applied at rate of 1 gallon/sq. ft (3.785 liters/30.48 cm²) on 11/5/69. (Source, Millcreek Pilot Research Plant, Cincinnati, Ohio).

Fecal coliforms per gallon 1.09 x 10⁸ (2,800,000/100 ml)

Ps. aeruginosa per gallon 41,600 (1,100/100 ml)

Salmonella sp. per gallon 2,800 (74/100 ml)

Plot 3: Undigested secondary activated sludge 4% conc. solids
applied at rate of 1 gallon/sq. ft (3.785 liters/30.48
cm²) on 11/16/69. (Source, Millcreek Pilot Research
Plant, Cincinnati, Ohio, 11/13/69).

Fecal coliforms per gallon 7.57 x 10⁸ (20,000,000/100 ml)

Ps. aeruginosa per gallon 75,700 (2,000/100 ml)

Salmonella sp. per gallon 352,000 (9,300/100 ml)

SAMPLING METHOD

Soil samples from the test areas were obtained by using a sharpened cork borer (alcohol flamed) with a diameter of approximately 1/2 inch (1.25 cm), and boring into the ground approximately 2 inches (5 cm), and extruding core samples into sterile containers. The samples were taken immediately to the laboratory, and 15 grams of soil sample weighed out on sterile weighing papers, and placed in 150 ml of pH 7.2 standard dilution water, with sterile glass beads in screw cap bottles. The sample was shaken vigorously, and the pathogens determined by the multiple tube method using 5-tube with 3- 5 dilutions as necessary, as described by Kenner (6). The fecal coliforms were determined by testing the supernatant after the soil had settled for 5- 10 minutes, by the M-FC method. Each 10 ml of soil-dilution water was equivalent to 1 gram of soil.

RESULTS

Table 1 presents the results of bacteriological assay of soils from three plots of grass-covered ground that had been treated with: (1) anaerobically digested primary sludge, (2) undigested activated sludge, and (3) undigested activated sludge that had been concentrated until 4% solids was obtained. The numbers per gram, admittedly, appear small; however, a gram of soil could be described as being as large as a "pinch" of a substance. The numbers of pathogens actually determined were for 10 grams of soil, and reduced one decimal point for comparison to the fecal coliform results. Of interest are the maximum number of days that the indicator organisms and the two pathogens persisted. The weather in the period from mid-December 1969 to March 1970 was one of severe cold with record snowfalls for this area, so that much freezing and thawing was experienced, along with record precipitation.

Table 1. Summary of Pecol Coliform, Ps. aeruginosa, and Salmonella sp. Isolates. Wt Gram of Sludge-Treated Soil at Weekly Intervals

Weather Bureau Reports During Study														
Anasr. Dig. Primary Sludge Treated				Undigested Activated Sludge (48 Conc.) Treated				Undigested Activated Sludge (48 Conc.) Treated						
Plot 1				Plot 2				Plot 3						
Date	FC	Ps.	Salin.	Weeks	FC	Ps.	Salin.	Weeks	High °F	High °C	Low °F	Low °C	Total Precip. inches	Total Precip. cm
11/ 6/69	530	<0.18	<0.18	0	4500	0.45	0.45		60	15.6	33	0.6	1.31	3.33
11/12/69	6100	<0.18	0.2	1	6800	1.5	0.78		58	14.4	21	- 6.1	0.52	1.32
11/17/69	500	<0.18	0.2	2	5000	0.4	0.68		55	12.8	18	- 7.8	2.04	5.18
11/24/69	120	0.4	<0.18	3	900	0.7	<0.18		58	14.4	28	- 2.2	0.3	0.76
12/ 1/69	670	<0.18	<0.18	4	500	2.0	<0.18		42	5.6	27	- 2.8	0.4	1.02
12/ 8/69	80	0.2	<0.18	5	95	4.3	<0.18		3	48	8.9	18	- 7.8	4.47
12/15/69	210	<0.18	<0.18	6	390	0.78	<0.18		4	47	8.3	24	- 4.4	0.08
12/22/69	425	<0.18	<0.18	7	260	0.92	<0.18		5	39	3.9	19	- 7.2	26.16
12/29/69	90	0.45	0.18	8	400	0.35	0.45		6	33	0.6	17	- 8.3	1.93
1/ 5/70	270	<0.18	<0.18	9	850	0.45	0.20		7	32	0	- 3	-19.4	0.02
1/12/70	50	<0.18	<0.18	10	150	0.20	0.20		8	41	5.0	2	-16.7	1.30
1/19/70	6.7	<0.18	<0.18	11	100	0.20	<0.18		9	48	8.9	- 2	-19.0	0.30
1/26/70	< 3.3	<0.18	<0.18	12	65	0.65	<0.18		10	65	18.3	- 2	-19.0	0.95
2/ 2/70	< 3.3	0.36	<0.18	13	3.3	0.74	<0.18		11	50	10.0	- 1	-18.3	3.28
2/ 9/70	< 3.3	0.18	<0.18	14	17	<0.18	<0.18		12	41	5.0	18	- 7.8	4.39
2/16/70	< 2.0	<0.18	<0.18	15	8	<0.18	<0.18		13	62	16.7	14	-10.0	0
2/24/70	0.0	0.36	<0.18	16	14	0.93	0.20		14	56	13.3	10	-12.2	0
3/ 2/70	0.0	0.36	<0.18	17	8	0.18	<0.18		15	63	17.2	35	1.7	2.03
3/16/70	0.0	<0.18	<0.18	19	17	0.20	<0.18		16	57	13.9	16	- 8.9	11.82
3/30/70	1.0	<0.18	<0.18	21	2	1.70	<0.18		17	57	13.9	26	- 3.3	3.0
4/27/70	0.0	<0.18	<0.18	23	0	<0.18	<0.18		19	78	25.6	29	- 1.7	7.62
6/ 8/70	8.0*	1.2*	<0.18	31	0	1.20*	<0.18		23	91	32.8	36	2.2	6.65
									29					22.02

Salmonella serotypes from soil 2 ident.; strains 8 identified

Salmonella serotypes from soil 15 ident.; strains 167 ident. 10 weeks S. derby (6) 11 weeks S. cubana (6)

Salmonella serotypes from soil 6 ident.; strains 49 identified 16 weeks S. derby (6)

Salmonella serotypes from soil 15 ident.; strains 167 ident. 10 weeks S. derby (6) 11 weeks S. cubana (6)

Maximum No. days detected 147 119 56

* Probably due to mowing

Table 2 lists the Salmonella serotypes found in each of the three sludge samples. It is amazing that so many different serotypes of Salmonella are present in less than 100 ml of sludge. As indicated, by astericks, are serotypes isolated from the sludge-treated soil samples during the course of the study.

Table 2. Salmonella serotypes Detected in
Sludges Used for Soil Spreading Experiments

Anaerobic digested primary sludge (11/5/69) used on Plot 1	Undigested activated sludge (11/5/69) used on Plot 2	Undigested activated sludge (Conc. 4% solids) Plot 3
1. S. chester (B)*	1. S. anatum (B1)*	1. S. anatum (B1)
2. S. derby (B)	2. S. braenderup (C1)	2. S. blockley (C2)*
3. S. havana (G)	3. S. derby (B)*	3. S. braenderup (C1)*
4. S. sims bury (B4)	4. S. enteritidis (D)*	4. S. brancaster (B)
5. S. typhimurium (B)*	5. S. kottbus (C2)	5. S. californica (B)
	6. S. manhattan (C2)	6. S. chester (B)*
	7. S. schwarzengrund (B)*	7. S. cubana (G)*
	8. S. tshiongwe (C2)*	8. S. derby (B)*
	9. S. typhimurium (B)*	9. S. enteritidis (D)*
		10. S. haifa (B)*
		11. S. heidelberg (B)
		12. S. infantis (C1)
		13. S. kottbus (C2)*
		14. S. livingstone (C1)
		15. S. manhattan (C2)
		16. S. montevideo (C1)*
		17. S. newlands (B1)
		18. S. oranienburg (C1)
		19. S. sandiego (B)*
		20. S. schwarzengrund (B)*
		21. S. tshiongwe (C2)*
		22. <u>S. typhimurium (B)</u>
		23. Arizona*

* Serotypes isolated from soil samples during the study

DISCUSSION

From the results obtained in these studies it is evident that sludge-borne pathogens may persist on agricultural or grass land for at least 21 weeks. It is evident that sludges such as those in Table 2 are an accumulation of many *Salmonella* serotypes, and a source of nutrients for the growth of *Ps. aeruginosa*.

Some may question the inclusion of *Ps. aeruginosa* in this study for pathogens; however, this increasingly important (antibiotic-resistant) human and animal pathogen has also been described as a plant pathogen by Elrod and Braun (4).

Law (7) (page 29) states that incorporation of sludge into soil "at least 3 months" ahead of planting time leads to destruction of pathogenic organisms. The results of our study showed a maximum survival time of 112 days or 16 weeks for *Salmonella* sp. and 147 days or 21 weeks for *Ps. aeruginosa*, despite a most severe winter, with record precipitation of snow and rainfall, and despite the fact that all sampling was only from the top two inches of grass and soil.

III. ANALYSIS OF WASTE TREATMENT SLUDGES FOR SALMONELLA SPECIES AS A SURVEILLANCE TOOL

INTRODUCTION

Salmonellosis apparently is an endemic infection throughout the world. From results obtained in this study, it is a certainty that passive carriers of salmonella outnumber, by far, clinically reported cases in human beings.

Problems associated with complete surveillance of salmonella in any one locale, such as a large urban area, are due to many factors, such as: (1) lack of communication between interested health departments, (2) lack of reporting cases by local physicians, (3) use of antibiotics without accompanying laboratory diagnosis, (4) many diarrheal cases do not seek treatment, and (5) mistaken notion that large numbers of laboratory personnel and much expensive equipment is necessary to completely identify the most common serological types of salmonella.

It is suggested that it is possible to have a more complete and purposeful surveillance of salmonellosis for any urban area by the simple convenient method of monthly analysis of municipal treatment plant sludges.

In three CDC Surveillance reports (2), (3), and (4), the maximum numbers of salmonella serotypes reported in any one month for the entire State of Ohio was 17. In the analysis of one sample of concentrated, undigested, activated sludge from the Millcreek Treatment Plant, Cincinnati, Ohio for November 1969, 23 serotypes of salmonella, and Arizona were detected. The treatment plant serves the western, northern, and core areas of the Metropolitan Sewer District of Greater Cincinnati.

RESULTS

Table 1 summarizes the analysis of various types of municipal sludges, by a method devised by Kenner (1). The results for April 1969 were obtained by analysis of one sample of anaerobically-digested primary sludge from the Millcreek Treatment Plant, and two samples of anaerobically digested primary sludge from the smaller Little Miami Treatment Plant serving the eastern portion of Cincinnati. The results for May 1969 are from secondary anaerobically digested primary sludge (ready for incineration) and its supernatant portion. The August 1969 results are from a single Millcreek undigested activated sludge and treatment effluent. The most productive sludge was undigested, activated sludge (November 1969) in which the solids were allowed to settle to a 4% concentration.

Table 1. Summary of Salmonella Serotypes Isolated from Various Types of Municipal Wastewater Treatment Plant Sludges

Serotype	Group	April 1969 ¹	May 1969 ²	August 1969 ³	Nov. 1969 ⁴	Rank*
anatum	B1	MC		MC	MC	15
binza	B2			MC		
blockley	C2	MC	LM		MC	8
braenderup	C1	LM			MC	24
brancaster	B				MC	
california	B				MC	
chester	B	MC&LM	LM	MC	MC	23
Cl:eh:l(L)	C1	MC				
cubana	G	MC		MC	MC	
derby	B	LM			MC	13
drypool	B2			MC		
enteritidis	D	LM	LM		MC	3
friedenau	G			MC		
give	B1	LM		MC		
haifa	B				MC	
heidelberg	B	MC			MC	2
infantis	C1	MC&LM	LM	MC	MC	5
javiana	D		LM			12
kottbus	C2	LM		MC	MC	
lexington	B1			MC		
livingstone	C1				MC	
lomita	C1	LM				
manhattan	C2	LM	LM		MC	16
montevideo	C1				MC	11
muenchen	C2		LM			18
newlands	B1				MC	
newport	C2	MC&LM			MC	4
norwich	C1	MC				
oranienburg	C1				MC	10
paratyphi B	B	LM	LM			20
saint paul	B			MC		6
san diego	B	MC&LM			MC	21
schwarzengrund	B				MC	
senftenberg	B4	LM		MC		
thompson	C1			MC		9
tshingwe	C2				MC	
typhimurium	B	MC&LM	LM		MC	1
Arizona	-				MC	

No. of
serotypes 37 Total
No. strains 702 Total

20 9 13 23
269 84 65 284

- 1 Millcreek Treatment Plant anaer. digested prim. sludge (4/7/69); Little Miami Treatment Plant anaer. digested primary sludge (4/9 - 4/14/69)
- 2 Little Miami Treat. Plant Rec. anaer. dig. primary sludge and supernatant (5/5/69) - 1 sample
- 3 Millcreek undigested activated sludge and effluent - Pilot Research Plant (8/12/69) 8/7/69) respectively.
- 4 Millcreek undigested act. sludge conc. 4% solids (11/13/69) - 1 sample
- * Rank as human enteric path. 1967 CDC Salmonella Surv., Martin & Bwing (5).

DISCUSSION

The urban area served by the two treatment plants described is approximately 100 square miles with a population of a little over 500,000, as compared to the entire State of Ohio with an area exceeding 41,000 square miles and a population of approximately 10,500,000. Therefore, if the entire state reports only 65 clinical cases represented by only 17 serotypes, and one sample of sludge contains 23 serotypes, evidently the usefulness to local urban areas of sludge analysis would be greater to concerned public health officials.

An example of the usefulness of such local reports by urban laboratories can be obtained from local experience. In October 1970, in a monthly report by our laboratory, the isolation of 7 strains of hog cholera bacteria (S. cholerae-suis var. kunzen-dorf) from domestic sewage from the east part of Cincinnati went unnoticed. At the same time it was front-page news that nearby Clinton County, Ohio was experiencing a hog cholera epidemic. No human clinical cases were reported in Ohio in October or November 1970 Surveillance Reports. Either human carriers were present, or contaminated pork products were being sold in this area of Cincinnati.

Most surveillance reports give results of disease occurrence "after the fact," reporting accumulated data from State Health Departments, from widely scattered locations within the state. Often the results are reported months later. That these reports can be of help to local health boards in large urban areas is questionable.

It is suggested that a monthly assay of municipal sludges from defined localities in urban areas could "point out" a high carrier rate of certain serological types of Salmonella currently, or the presence of those serological types in local food or feed products that need inspection.

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REFERENCES

I.

1. Bonde, G. J. 1966. Bacteriological methods for estimation of water pollution. Health Lab. Sci., 3:124-128.
2. Brezenski, F. T., R. Russomanno, and P. DeFalco, Jr. 1965. The occurrence of salmonella and shigella in post-chlorinated and non-chlorinated sewage effluents and receiving waters. Health Lab. Sci., 2:40-47.
3. Brezenski, F. T., and R. Russomanno. 1969. The detection and use of salmonellae in studying polluted tidal estuaries. J. Water Poll. Control Fed., 41:725-737.
4. Condit, P., and E. Stone. 1965. Water-borne Salmonella typhimurium, Riverside, California. National Communicable Disease Center, PHS: Morbidity and Mortality Weekly Report, 14:185-186; 194-195.
5. Edwards, P. R., and W. H. Ewing. 1966. Identification of Enterobacteriaceae, 2nd ed. Burgess Publishing Company, Minneapolis.
6. Fair, J. F., and S. M. Morrison. 1967. Recovery of Bacterial pathogens from high quality surface waters. Water Resources Research, 3:799-803.
7. Fair, J. F., and S. M. Morrison. 1968. Reply. Water Resources Research, 4:452.
8. Ino, J., and C. D. Graber. 1955. Recovery of Salmonella from contaminated cultures. U. S. Armed Forces Med. J., 6:586-587.
9. Kabler, P. 1959. Removal of pathogenic microorganisms by sewage treatment processes. Sewage and Indust. Wastes, 31:1373-1382.
10. Kabler, P. 1962. Reduction of pathogenic microorganisms by sewage treatment. Environ. Health (India) 4:258-268.
11. Kenner, B. A., S. W. Rockwood, and P. W. Kabler. 1957. Isolation of members of the genus Salmonella by membrane filter procedures. Appl. Microbiol., 5:305-307.

12. King, E. O., M. K. Ward, and D. E. Raney. 1954. Two simple media for the demonstration of pyocyanin and fluorescein. *J. Lab. Clin. Med.*, 44:301-307.
13. King, S., and W. I. Metzger. 1968. A new plating medium for the isolation of enteric pathogens. I. Hektoen enteric agar. *Appl. Microbiol.*, 16:577-578.
14. Livingstone, D. J. 1965. An improved method for isolating salmonellae from polluted waters. *Public Health (Johannesburg)*, 65:87-88.
15. Mackenzie, C. R., and D. J. Livingstone. 1968. Salmonellae in fish and food. *South Africa Med. J.*, 42:999-1003.
16. Martin, J. D. 1957. Zoonoses in the South. *Public Health Rep.*, 72:210-216.
17. Martin, W. J., and W. H. Ewing. 1969. Prevalance of serotypes of Salmonella. *Appl. Microbiol.*, 17:111-117.
18. McCullough, N. B., and C. W. Eisele. 1951. Experimental human Salmonellosis. I. Pathogenicity of strains of Salmonella meleagridis and Salmonella anatum obtained from spray-dried whole egg. *J. Infec. Dis.*, 88:278-289.
19. Moore, B. 1954. Sewage contamination of coastal bathing waters. *Bull. Hygiene*, 29:689-704.
20. Moore, B. 1957. Observations pointing to the conjunctive as the portal of entry in salmonella infection in guinea pigs. *J. Hyg., (Cambridge)* 55:414-433.
21. Morgan, G. B., P. Gubbins, and V. Morgan. 1965. A critical appraisal of the membrane filter technic. *Health Lab. Sci.*, 2:227-237.
22. Raj, H. 1966. Enrichment medium for selection of Salmonella from fish homogenate. *Appl. Microbiol.*, 14:12.
23. Read, R. B. Jr., and A. L. Reyes. 1968. Variation in plating efficiency of salmonellae on eight lots of brilliant green agar. *Appl. Microbiol.*, 16:746-748.
24. Reitler, R., and R. Seligmann. 1957. Pseudomonas aeruginosa in drinking water. *J. Appl. Bacteriol.*, 20:145-150.

25. Smith, H. H., and C. Anderson-Langmuir. 1966. Variable results from triple sugar iron agar in screw-capped tubes. Tech. Bull. Reg. Med. Technologists, 36:218-221.
26. Spino, D. F. 1966. Elevated-temperature technique for isolation of Salmonella from streams. Appl. Microbiol., 14:591-596.
27. Stuart, P. F., and H. Pivnick. 1965. Isolation of Salmonellae by selective motility systems. Appl. Microbiol., 13:365-372.
28. Taylor, W. I. 1965. Isolation of Shigellae I. Xylose lysine agars; new media for isolation of enteric pathogens. Tech. Bull. Reg. Med. Technologists, 35:161-165.
29. Taylor, W. I., and D. Schelhart. 1967. Isolation of Shigella IV. Comparison of plating media with stools. Tech. Bull. Reg. Med. Technologists, 37:222-228.
30. Taylor, W. I., and D. Schelhart. 1968. Isolation of Shigellae V. Comparison of enrichment broths with stools. Appl. Microbiol., 16:1383-1386.
31. Tidwell, W. L., C. D. Heather, and C. Merkele. 1955. An autoclave sterilized medium for the detection of urease activity. J. Bacteriol., 69:701-702.
32. Walton G., and G. L. Culp. 1963, 1964. Chlorine disinfection in primary sewage treatment. A review of the literature. Parts 1 through 4. Water and Sewage Works, 110:415; 110:438-439; 111:37-38; 111:80-81.
33. Wolman, M. G. 1968. Comments on "Recovery of bacterial pathogens from high quality surface water" by J. F. Fair and S. M. Morrison. Water Resources Research, 4:451.

II.

1. Anderson, M. S., 1956. Comparative analysis of sewage sludges. Sewage and Ind. Wastes, 28:132-135.
2. Cooke, W. B., 1969. Fungi in soils over which digested sewage sludge has been spread. Mycopath. et Mycol. Appl., 39:209-229.

3. Dotson, G. K., and J. O. Evans, 1969. Occurrence and survival of indicator organisms in sludge-treated soils. Internal Report, AWTRL, Cincinnati, Ohio.
4. Elrod, R. P., and A. Braun, 1942. Pseudomonas aeruginosa; its role as a plant pathogen. J. Bacteriol., 44:633-644.
5. Jackson, L. W., 1947. Sewage plant sells sludge and effluent. Engr. News-Record, 139:56-58.
6. Kenner, B. A., 1971. Simultaneous quantitation of Salmonella species and Pseudomonas aeruginosa I. Polluted waters. This Publication.
7. Law, J. P., 1968. Agricultural utilization of sewage effluent and sludge. An annotated bibliography. Fed. Water Poll. Control Administration, U. S. Dept. of the Interior. CWR-2.
8. Merz, R. C., 1959. Utilization of liquid sludge. Water and Sewage Wks., 106:489-493.

III.

1. Kenner, B. A., 1971. Simultaneous Quantitation of Salmonella species and Pseudomonas aeruginosa. I. Polluted Waters. This Publication.
2. National Communicable Disease Center Salmonella Surveillance, Dec. 1968, Report No. 79.
3. Center for Disease Control Salmonella Surveillance, March 1971, Report 103.
4. Center for Disease Control Salmonella Surveillance, March 1971, Report 104.
5. Martin, W. J., and W. H. Ewing, 1969. Prevalence of serotypes of Salmonella. Appl., Microbiol., 17:111-117.